Properties of Heparan Sulphate and Chondroitin Sulphate from Young and Old Human Aortae

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1. Glycosaminoglycans were liberated from old and young human ascending aortae by digestion with papain. Heparan sulphate and chondroitin sulphate were separated by the different solubilities of their complexes with cetylpyridinium chloride in solutions of sodium chloride. Final fractionation was achieved by salt-gradient column chromatography on Dowex 1 (Cl⁻ form). 2. Heparan sulphate from old aortae showed a slight, but consistent, increase in sulphation compared with heparan sulphate from young aortae. 3. The major amino acids associated with aortic heparan sulphate and chondroitin sulphate were serine, glycine, glutamic acid and aspartic acid. Heparan sulphate and chondroitin sulphate from old aortae contained about twice as much total amino acid as heparan sulphate and chondroitin sulphate from young aortae. Alkali hydrolysis resulted in the destruction of more serine in chondroitin sulphate from old, compared with young, aortae. 4. Molecular weights of glycosaminoglycans from old and young aortae were found to be similar, and in the region of 35 000. 5. It is suggested that there is an increased degree of protein-glycosaminoglycan cross-linking in old aortae.

Three major groups of glycosaminoglycans, hyaluronate, heparan sulphate and chondroitin sulphate, have been found in human arterial tissue (Kaplan & Meyer, 1960). Their relative proportions have been found to alter with age (Kaplan & Meyer, 1960), blood pressure (Manley, 1965) and anatomical site (Manley & Hawksworth, 1965).

Glycosaminoglycans of connective tissue are attached to a protein core (Shatton & Schubert, 1954) from which they may be liberated by proteolytic digestion. A residual peptide fragment is usually associated with glycosaminoglycans prepared in this way. In a preliminary communication Manley & Mullinger (1967*a*) suggested that glycosaminoglycans liberated from old human aortae by proteolytic digestion contained more residual peptide than those liberated in the same way from young arterial tissue.

In the present study the chemical and physical properties of glycosaminoglycans from young and old human aortae were investigated in an attempt to elucidate the changes that occur with age. Human aortae develop an increasing degree of atherosclerosis with age, which complicates the phenomenon of aging. Therefore a localized segment of the ascending aorta, which is relatively unaffected by atherosclerosis, was chosen for study.

MATERIALS AND METHODS

Material. The two age groups selected for study were 20 ± 5 years (young) and 80 ± 5 years (old). The ascending aorta between 1.0 and 5.0 cm. above the aortic valve was obtained at necropsy, and the fibrous adventitia were removed. The remaining intima-media preparation was diced and dried *in vacuo* over silica gel.

Crude papain was obtained from British Drug Houses Ltd. (Poole, Dorset) and twice-recrystallized papain was prepared from this as described by Kimmel & Smith (1954). The concentration of the enzyme was determined as described by Whitaker & Bender (1965) and its activity determined with benzoylarginine amide ethyl ester as substrate (Schwert & Takenaka, 1956). Ovine testicular hyaluronidase and cow pancreas deoxyribonuclease were obtained from British Drug Houses Ltd. Visking dialysis tubing was obtained from Hudes Merchandising Corp. Ltd. (London W.1).

Isolation of glycosaminoglycans. Young and old tissue was processed in parallel. Samples (6g.) were digested under N₂ in 600 ml. of 0.1 M-sodium phosphate buffer, pH 6.5, containing 5 mM-EDTA, 5 mM-cysteine hydrochloride and 60 mg. of crystalline papain, as described by Manley (1965). The activity of the papain used was the same in all preparations. A parallel control digestion was carried out with small samples of old and young arterial tissue, portions being withdrawn at intervals for determination of the activity of the papain. After incubation for 24 hr. at 60°, the digests were dialysed in Visking tubing

against water for 48 hr. at 4°. The samples were concentrated to 200 ml. by exposing the Visking tubing to a forced draught. Then NaCl was added to give a concentration of 0.04 M, and 200 ml. of 0.1% CPC* in 0.04 M-NaCl was added slowly with stirring. After 24 hr. at 20° the precipitates were collected by centrifugation at 1500g for 1 hr. The precipitates were dissolved in 40 ml. of 2 M-NaCl, and 400 ml. of ethanol was added slowly with stirring. The precipitates that had formed after 48 hr. at 4° were collected by centrifugation at 1500g for 30 min. The precipitates were washed with ethanol and dissolved in 40 ml. of water. The samples were dialysed against water for 48 hr. at 4°. DNA was removed from the samples by digestion with 10 mg. of cow pancreas deoxyribonuclease in 100 ml. of 0.02 M-tris-HCl buffer, pH 7.9, containing 0.02 M-MgCl₂. Digestion was carried out for 24 hr. at 37°. The samples were then dialysed against water for 48 hr. at 4°, and the glycosaminoglycans were precipitated by addition of ethanol to 90% (v/v) final concentration. The precipitates were collected by centrifugation, dissolved in 40 ml. of water and dialysed against water for 48 hr. at 4°.

Separation of glycosaminoglycans. The glycosaminoglycans were precipitated by the addition of 10% (w/v) CPC and partially fractionated according to the solubility of the precipitate in NaCl solutions as described by Schiller, Slover & Dorfman (1961). Each fraction was then subjected to chromatography on a 2 cm. × 45 cm. column of Dowex 1 (X2; Cl⁻ form; -400 mesh) by a modification of the method described by Schiller *et al.* (1961). The column was eluted at 8 ml./hr. with a steadily increasing concentration of NaCl, rising from 0.1 M to 2.5 M. Effluent fractions containing glycosaminoglycans were located by uronic acid assay. Pooled fractions were dialysed against water for 48 hr. at 4° and concentrated on a rotary evaporator. Samples were examined by electrophoresis on cellulose acetate.

Amino acid analysis. Glycosaminoglycan samples (4 mg.) were hydrolysed in vacuum-sealed tubes at 110° for 17 hr. in twice-distilled constant-boiling HCl. After hydrolysis the HCl was removed *in vacuo* over KOH pellets. Alkali-treated samples were subsequently placed on a 1 cm. × 8 cm. column of Dowex 50 (X8; H⁺ form; -400 mesh) and eluted with 50 ml. of 5 M-NH₃. The effluent was dried on a rotary evaporator. Amino acids were determined on a Technicon AutoAnalyzer. Because of the limited amount of material available, single amino acid analyses were carried out on each sample, and no correction was made for decomposition during hydrolysis.

Alkali hydrolysis. Samples (10 mg./ml.) were treated with 0.5 M-NaOH for 36 hr. at room temperature.

Electrophoresis. Samples (10 μ l.) containing 10–20 μ g. of glycosaminoglycan were applied to 5 cm. × 20 cm. strips of cellulose acetate (Oxoid Ltd., London E.C.4). Electrophoresis was carried out in Michaelis veronal-acetate buffer, pH 9.2, at a constant potential gradient of 20 v/cm. for 80 min. at 20°. Glycosaminoglycans were located by staining with Alcian Blue and measured by reflexion densitometry as described by Manley (1965). For a careful comparison of the mobilities of heparan sulphate from old and young aortae, 1 μ l. samples containing 1 μ g. of glycosaminoglycan were applied as small spots. Heparan sulphate from young aortae was applied with heparan sulphate from old aortae on either side, and vice versa.

* Abbreviation: CPC, cetylpyridinium chloride.

Determination of dermatan sulphate. The proportion of dermatan sulphate in preparations of chondroitin sulphate was determined by the method of Schmidt & Dmochowski (1964a). Samples containing 400 μ g. of uronic acid were treated with hyaluronidase, then applied to a 1 cm. × 100 cm. column of Sephadex G-50 (fine grade) (Pharmacia Ltd., London W.13). Material was eluted with 0.5 M-NaCl at 6.0 ml./hr. Fractions were collected after 3½ hr. at 10 min. intervals. The uronic acid content of the fractions was determined by the method of Bitter & Muir (1962) modified for the Technicon AutoAnalyzer. Undigested samples of chondroitin sulphate containing 70 μ g. of uronic acid were also applied to the column.

Other analytical methods. Hexosamine was measured by the method of Boas (1953). Hydroxyproline was determined as described by Neuman & Logan (1950). Hexuronic acid was measured by the method of Bitter & Muir (1962) with glucuronolactone (Sigma Chemical Co., St Louis, Mo., U.S.A.) as standard. Phosphate was determined as described by Bartlett (1959). Protein was determined by the micro biuret method of Itzhaki & Gill (1964). Sulphate was measured by hydrolysing samples in 50 μ l. of 30% (v/v) formic acid at 100° for 8 hr. as described by Muir & Jacobs (1967). A 25 μ l. portion of 0.2% sodium acetate was added and the samples were dried *in vacuo* over KOH pellets. Water (50 μ l.) was added and the sulphate determined by the barium chloroanilate method of Spencer (1960) scaled up to analyse 50 μ l. samples containing 1–5 μ g. of sulphate.

Determination of concentration. The concentrations of four solutions were determined by drying samples to constant weight over P₂O₅ *in vacuo*. These solutions were used to determine the specific refractive increment with a Brice-Phoenix differential refractometer. The concentrations of all other solutions were obtained by differential refractometry.

Determination of partial specific volume. The partial specific volume of each sample was determined by comparing the density of a solution with that of its diffusate. Densities were determined in a density gradient of bromobenzene and paraffin as described by Schachman (1957).

Determination of optical rotation. The specific rotation was determined at 25° and 589 nm. with a Polaromatic 62 polarimeter (Bellingham and Stanley Ltd.).

Determination of i.r. spectra. A 600 μ g. portion of the sample was mixed with 30 mg. of KCl and the i.r. spectrum was determined with a Perkin-Elmer 125 diffraction-grating spectrophotometer.

Analytical sedimentation. All solutions were dialysed against 0.5 M-NaCl before sedimentation. Sedimentation-velocity experiments were carried out at 59780 rev./min. in a Beckman Spinco model E ultracentrifuge with schlieren optics.

Columns of solution (3 mm.) were sedimented to equilibrium at speeds between 9900 and 16200 rev./min. for 16 hr. and the resulting concentration distributions determined by using both interference and schlieren optics. The hinge point was determined from the interferograms by means of the achromatic fringe (Richards & Schachman, 1959), and the weight-average molecular weight, M_w , was determined from the slope, at the hinge point, of a plot of $\log c$ versus r^2 , where c is the concentration at distance r from the centre of rotation. The z-average molecular weight, M_z , was determined from the schlieren pictures by the method of Lamm (1929).

RESULTS

The total yield of glycosaminoglycans in terms of hexuronic acid was 27.3mg./g. dry wt. of tissue from young aortae and 24.8mg./g. dry wt. of tissue from old aortae. Assays of papain activity in control digests showed that about 50% of the original activity remained after 24hr. incubation. At no time did the activity differ significantly in digests of old and young aortae.

After deoxyribonuclease digestion, young and old aortic glycosaminoglycans contained less than 0.06% of phosphate, indicating that contamination with nucleic acids was negligible. Preliminary fractionation of the isolated glycosaminoglycans by using the differential solubility of the CPC complex in sodium chloride solutions gave results that differed from some previous reports. The aortic hyaluronate-CPC complex was soluble in 0.4M-sodium chloride, the heparan sulphate-CPC complex was soluble in 0.6M-sodium chloride and the chondroitin sulphate-CPC complex was soluble in 1.5M-sodium chloride. Schiller *et al.* (1961) found that the CPC complexes of heparan sulphate and chondroitin sulphate derived from rat skin were soluble in 1.2M-sodium chloride, but Muir (1965) noted that the CPC complex derived from aortic heparan sulphate was eluted from a cellulose column at a notably low (0.6M) concentration of potassium chloride.

Electrophoresis on cellulose acetate of the samples obtained by preliminary CPC fractionation showed some cross-contamination of the three fractions. The second fractionation, by salt-gradient column chromatography on Dowex 1 (Cl⁻ form), yielded fractions that moved as single peaks on cellulose acetate electrophoresis (Fig. 1) and in the ultracentrifuge.

The uronic acid-containing material eluted from the column of Dowex 1 (Cl⁻ form) in 1.6M-sodium chloride contained approximately equimolar proportions of hexosamine, uronic acid and sulphate. The amino acid chromatogram showed that the hexosamine was galactosamine (99%) with a small contamination (1%) of glucosamine. The electrophoretic migration on cellulose acetate under the conditions described was 0.38cm./hr./v/cm., and identical with that of a standard sample of chondroitin 6-sulphate (gift from Dr R. W. Jeanloz). The optical rotation, $[\alpha]_D^{25}$, was $-20.8 \pm 1.0^\circ$ for the sample obtained from young aortae and $-18.2 \pm 0.8^\circ$ for the sample obtained from old aortae. The values are within the range described for chondroitin 6-sulphate by Meyer, Davidson, Linker & Hoffman (1965). The i.r. spectra of the material derived from both young and old aortae showed, in the range 700–1000cm.⁻¹, peaks at 924, 850, 816, 770 and 712cm.⁻¹. A peak at 840cm.⁻¹, characteristic of

Fig. 1. Chromoscan traces of glycosaminoglycans from young human aorta, separated by electrophoresis on cellulose acetate and stained with Alcian Blue. Electrophoretic migration was from left to right. Technical details are given in the text. (a) Crude preparation of glycosaminoglycans prepared by papain digestion, CPC precipitation, NaCl dissociation and final precipitation in ethanol; peak 1 corresponds in mobility with hyaluronic acid, peak 2 with heparan sulphate and peak 3 with chondroitin sulphate. (b) Material eluted from a Dowex 1 (Cl⁻ form) column by 1.6M-NaCl. (c) Material eluted from a Dowex 1 (Cl⁻ form) column by 0.9M-NaCl.

dermatan sulphate (Mathews, 1958), was not observed. On the basis of these data, summarized in Table 1, the material was designated as chondroitin sulphate.

Table 1. *Chemical composition of glycosaminoglycan samples from young and old aortae*

	Heparan sulphate		Chondroitin sulphate	
	Young aortae	Old aortae	Young aortae	Old aortae
Hexuronic acid (μ moles/mg.)	2.05	2.07	1.56	1.43
Hexosamine (μ moles/mg.)	1.68	1.69	1.62	1.45
Sulphate (μ moles/mg.)	0.61	0.71	1.59	1.48
Optical rotation ($[\alpha]_D^{25}$)	$+70.2 \pm 3.5^\circ$	$+63.3 \pm 3.2^\circ$	$-20.8 \pm 1.0^\circ$	$-18.2 \pm 0.8^\circ$
Glucosamine/galactosamine ratio	1.0:0.009	1.0:0.010	0.008:1	0.009:1
Amino acids (μ moles/g.)				
Aspartic acid	20	43.5	12	16
Threonine	4.5	14.5	8	9
Serine	32	56.0	27	48.5
Glutamic acid	24.5	48.5	18	28
Proline	N.D.	N.D.	5	9
Glycine	26.5	65.5	23	37.5
Alanine	14.5	28.0	13	18
Tyrosine	N.D.	N.D.	3	11.5
Lysine	N.D.	N.D.	3.5	5

The major amino acids present were serine, glycine, glutamic acid, aspartic acid and alanine. About twice as much total amino acid was associated with the material obtained from old aortae, although the relative proportions of the amino acids were the same in the chondroitin sulphates obtained from young and old aortae. Mild alkali hydrolysis resulted in the destruction of 4.7μ moles of serine/g. of chondroitin sulphate from young aortae, and 12.2μ moles of serine/g. of chondroitin sulphate from old aortae.

Chondroitin sulphate preparations from old and young aortae were eluted from a column of Sephadex G-50 as a single peak with the void volume (Fig. 2). After digestion with testicular hyaluronidase, about 5% of the original material remained undigested and was eluted at the void volume. The remaining products of digestion were well separated from the undigested material, being eluted as three uronic acid-containing peaks between the void volume and the total volume. Since chondroitin 4-sulphate and chondroitin 6-sulphate are digested by testicular hyaluronidase, whereas dermatan sulphate is not (Schmidt & Dmochowski, 1964a), these results suggest that the contents of dermatan sulphate in the chondroitin sulphate preparations from young and old aortae were not measurably different, and were about 5%.

The uronic acid-containing material eluted from the column of Dowex 1 (Cl^- form) in 0.9M-sodium chloride contained more uronic acid, as measured by the carbazole reaction, than hexosamine. The presence of such a compound in aorta has been described by Muir (1961), who noted both the high

Fig. 2. Elution patterns of aortic chondroitin sulphate from a column of Sephadex G-50 before and after hyaluronidase digestion. ○, Undigested aortic chondroitin sulphate measured as uronic acid in column effluent; ●, aortic chondroitin sulphate after digestion with testicular hyaluronidase, measured as uronic acid in column effluent.

uronic acid content and the low sulphate content. The material had an electrophoretic mobility on cellulose acetate of 0.28 cm./hr./v/cm. under the conditions described. The optical rotation, $[\alpha]_D^{25}$, was $+70.2 \pm 3.5^\circ$ for the samples obtained from young aortae and $+63.3 \pm 3.2^\circ$ for the samples obtained from old aortae. The material was completely resistant to the action of testicular hyaluronidase.

On the basis of these data, summarized in Table 1, the material was designated a heparan sulphate of low sulphation.

The molar ratio of sulphate to hexosamine was found to be consistently higher in heparan sulphate from old aortae than in heparan sulphate from young aortae. In eight separate assays the sulphate content of heparan sulphate from old aortae was found to be 0.42 ± 0.02 mole/mole of hexosamine, and from young aortae 0.36 ± 0.01 mole/mole of hexosamine. This small difference in sulphation was also reflected in the electrophoretic mobility on cellulose acetate. In ten separate experiments, under carefully controlled conditions, heparan sulphate from young aortae moved $98.5 \pm 0.5\%$ of the distance moved by heparan sulphate from old aortae. Statistical analysis showed this difference to be highly significant ($P < 0.001$).

The major amino acids present were serine, glycine, glutamic acid, aspartic acid and alanine. As with the chondroitin sulphates, about twice as much total amino acid was associated with the material prepared from old aortae, although the relative proportions of the different amino acids were the same.

Insufficient hyaluronate was obtained from aortae to justify a detailed study. The relative proportions of chondroitin sulphate, heparan sulphate and hyaluronate in old and young aortae, as determined by salt-gradient column chromatography on Dowex 1 (Cl⁻ form), with uronic acid assay of the column effluent with glucuronolactone as standard, are shown in Table 2.

Table 2. *Relative proportions of glycosaminoglycans in young and old human aortae determined by salt-gradient column chromatography on Dowex 1 (Cl⁻ form) with uronic acid assay of the column effluent*

	Relative proportions (%)	
	Young aortae	Old aortae
Hyaluronate	2.4	1.9
Heparan sulphate	37.1	24.5
Chondroitin sulphate	60.5	73.4

Physical studies. The values of specific refractive increment, dn/dc , and of partial specific volume, $\bar{v}$, are given in Table 3. These values are fairly typical for polysaccharides, but much lower than the typical values for proteins.

The chondroitin sulphate and heparan sulphate from both young and old aortae all had sedimentation coefficients of 1.3×10^{-13} sec. at concentrations of 5 mg./ml. The coefficients were slightly dependent on concentration, but sufficient results have not yet been obtained for reliable extrapolations to zero concentration.

The plots of $\ln c$ versus r^2 for both heparan sulphate samples at sedimentation equilibrium were straight lines, but the apparent weight-average molecular weight, $M_w(\text{app.})$, was dependent on the concentration of the solution. A plot of the reciprocal of the apparent weight-average molecular weights versus concentration (Fig. 3) was linear, and by extrapolation to zero concentration yielded weight-average molecular weights of 35 000 for the sample from young aortae and 33 500 for the sample from old aortae.

The plots of $\ln c$ versus r^2 for the more highly charged chondroitin sulphates were slightly curved in the direction corresponding to non-ideality. The slopes were determined at the hinge points and the reciprocals of the apparent molecular weights plotted against the initial concentration of the solutions (Fig. 4). When extrapolated to zero concentration these plots indicated weight-average molecular weights of 35 000 for the chondroitin sulphate from young aortae and 34 000 for the material from old aortae.

The apparent z -average molecular weights were determined from the Lamm plots derived from the schlieren patterns. The reciprocals of the square root of these apparent z -average molecular weights were plotted against the initial concentrations of the solutions (Figs. 3 and 4) and extrapolated to zero concentration. The resulting values were 36 000 for the chondroitin sulphates from both young and old aortae and 45 000 for both the heparan sulphates.

These results are summarized in Table 3 and indicate that the chondroitin sulphates are very nearly homogeneous, since the z -average molecular weights are only very slightly greater than the

Table 3. *Physical properties of glycosaminoglycan samples*

	Heparan sulphate		Chondroitin sulphate	
	Young aortae	Old aortae	Young aortae	Old aortae
dn/dc (ml./g.)	0.136	0.136	0.136	0.134
$\bar{v}$ (ml./g.)	0.524	0.525	0.507	0.514
M_w	35 000	33 500	35 000	34 000
M_z	45 000	45 000	36 000	36 000
B (ml./g.)	143	155	322	377

Fig. 3. Variation of the apparent weight-average molecular weight, M_w (app.), and z-average molecular weight, M_z (app.), of heparan sulphate with the concentration, c , of the solution. ●, M_w (app.), for young heparan sulphate; ○, M_w (app.) for old heparan sulphate; ▲, M_z (app.) for young heparan sulphate; △, M_z (app.) for old heparan sulphate.

Fig. 4. Variation of the apparent weight-average molecular weight, M_w (app.) and z-average molecular weight, M_z (app.), of chondroitin sulphate with the concentration, c , of the solution. ●, M_w (app.) for young chondroitin sulphate; ○, M_w (app.) for old chondroitin sulphate; ▲, M_z (app.) for young chondroitin sulphate; △, M_z (app.) for old chondroitin sulphate.

weight-average values. The difference in weight-average molecular weight between the chondroitin sulphates from old and young aortae is probably within experimental error and so the two samples were of the same mass. However, the dependence of the apparent molecular weight on the concentration of the solution is slightly greater for the chondroitin sulphate from old aortae than for that from young aortae. The second virial coefficients, B , derived from the equation:

$$\frac{1}{M_w(\text{app.})} = \frac{1}{M_w} (1 + Bc)$$

are given in Table 3.

The z-average molecular weights of the heparan sulphates were appreciably higher than the weight-average values, indicating quite a large degree of

polydispersity. This should yield upward curvature of the plots of $\ln c$ versus τ^2 , compared with the downward curvature caused by non-ideality. It seems that the two effects are compensating one another, yielding the linear plots actually observed. This polydispersity seems to be slightly greater in the heparan sulphate from old aortae, since the weight-average molecular weight was slightly lower than that for the material from young aortae whereas the z-average molecular weight was the same. As for the chondroitin sulphates the second virial coefficient for the heparan sulphate from old aortae is slightly greater than that for the heparan sulphate from young aortae (Table 3).

DISCUSSION

The problems inherent in a study of the glycosaminoglycans of human aortae in the context of aging are considerable. The glycosaminoglycan content of aortic tissue is low; necropsy tissue from the age group 20 ± 5 years is scarce; the high incidence of atherosclerosis in the older age group, which in itself can produce complex changes in glycosaminoglycans (Manley & Mullinger, 1967b; Kumar, Berenson, Ruiz, Dalferes & Strong, 1967), means that a region of the aorta that remains unaffected by atherosclerosis must be selected for study. The small amount of pathologically significant material that can be obtained imposes strict limitations on the scope of biochemical studies that can be undertaken.

The total yield of glycosaminoglycans in terms of dry weight of aortic tissue was slightly lower from old than from young aortae. This is in agreement with the studies of Berenson (1961). The lower proportion of heparan sulphate and hyaluronate relative to chondroitin sulphate in old aortae confirms previous electrophoretic findings (Manley, 1965), but is at variance with the work of Schmidt & Dmochowski (1964b), who found a notably low proportion of heparan sulphate in young adult aortae. These workers used stepwise elution from columns of DEAE-sephadex, and in view of the low sulphate content of heparan sulphate from young aortae it is possible that some of the heparan sulphate from young, but not old, aortae was eluted with the hyaluronate fraction. The fact that the sulphate content of their heparan sulphate fraction (10.5%) is higher than that of their chondroitin sulphate fraction (6.6%) is difficult to explain.

No significant difference was found in the proportion of dermatan sulphate in old and young aortae, and this is contrary to the studies by Kaplan & Meyer (1960) and Schmidt & Dmochowski (1964b), who found a marked increase in dermatan sulphate with age. One possible explanation of this discrepancy is that these workers studied whole aortae,

which inevitably include an increasing degree of atherosclerosis with increasing age. An increase of dermatan sulphate has been found in atherosclerosis (Kumar *et al.* 1967). The material selected for the present study was entirely free of atherosclerosis.

Heparan sulphate isolated from old aortae was found to show a slight, but consistent, increase in the degree of sulphation when compared with material isolated by identical methods from young aortae. This small difference is supported by electrophoretic studies, where heparan sulphate from old aortae shows a slightly higher mobility, and by sedimentation-equilibrium studies in the ultracentrifuge, where heparan sulphate from old aortae shows a greater degree of non-ideality. The phenomenon was also noted, although not published, in heparan sulphates isolated from whole aortae (Manley & Mullinger, 1967*a*). The difference, although small, is consistent. It will be necessary to study the phenomenon in a large number of aortae of different ages before its significance in the context of ageing can be established. The observation of an increasing degree of sulphation in a connective-tissue glycosaminoglycan with increasing age is not without precedent, for Seno, Meyer, Anderson & Hoffman (1965) noted the occurrence of an over-sulphated keratan sulphate in old human costal cartilage.

The molecular weights of chondroitin sulphate and heparan sulphate were found to be higher than have been reported by some workers (Marler & Davidson, 1965; Knecht, Cifonelli & Dorfman, 1967). This may be due to the non-ideality of these highly charged molecules in ultracentrifugal sedimentation and osmotic-pressure studies, which makes it essential to extrapolate to zero concentration to obtain a true value. It should be mentioned that the small losses of glycosaminoglycans that inevitably occur during dialysis may result in selection of molecules of higher molecular weight for analysis.

It is noteworthy that the four major amino acids associated with both heparan sulphate and chondroitin sulphate in this study were serine, glycine, glutamic acid and aspartic acid. The four major amino acids associated with chondroitin sulphate isolated from bovine and human cartilage by Anderson, Hoffman & Meyer (1965) were serine, glycine, glutamic acid and proline. Very little proline was associated with chondroitin sulphate from human aorta. This may represent a tissue difference. A comparable replacement of proline by aspartic acid has been found in keratan sulphate isolated from cornea, whereas keratan sulphate isolated from cartilage, like chondroitin sulphate from cartilage, has proline as one of its major amino acids (Seno *et al.* 1965).

The major finding that has emerged from this

study is that there is more total amino acid associated with heparan sulphate and chondroitin sulphate liberated by proteolysis from old aortae than there is in material liberated by identical means from young aortae.

This phenomenon is unaffected by further exhaustive proteolysis. Manley & Mullinger (1967*a*) obtained similar results with material that had been successively redigested with papain and reisolated three times. This observation rules out the possibility that the results are due to incomplete proteolysis, and makes it unlikely that the results are due to differing degrees of proteolysis in young and old aortae. The observation of similar papain activities in digests of old and young aortic tissue is also against this possibility. One possibility that cannot be excluded by the present experiments is that peptide fragments from the papain itself may become firmly associated with the glycosaminoglycan chains and remain associated despite precipitation with CPC and subsequent salt-gradient column chromatography on Dowex 1 (Cl⁻ form). This unlikely event would not explain the difference between old and young glycosaminoglycans.

The glucosamine/galactosamine ratios for the samples of heparan sulphate and chondroitin sulphate suggest a minor degree of cross-contamination (about 1%). The phenomenon of an increased association of amino acids with material obtained from old aortae is, however, the same for both heparan sulphate and chondroitin sulphate, so that the possibility of a minor degree of cross-contamination is not important in this context.

The increased association of amino acids with glycosaminoglycans liberated by proteolysis from old aortae could be due either to more peptide being associated with the polysaccharide chains, or to shorter polysaccharide chains, in material isolated from old aortae.

The physical studies show no significant difference in the molecular weights of glycosaminoglycans isolated from old and young aortae. Thus it is unlikely that the polysaccharide chains are shorter in old aortae.

If more peptide is associated with polysaccharide chains from old aortae, it may be that a greater number of peptide chains are linked to the polysaccharide, or that the peptide chains are longer in material liberated from old aortae. From the amino acid analyses and weight-average molecular weights it can be deduced that there is approx. one serine molecule/glycosaminoglycan chain isolated from young aortae, and approx. two serine molecules/glycosaminoglycan chain isolated from old aortae.

The peptide-polysaccharide link of chondroitin sulphate isolated from cartilage is by xylosylserine (Muir, 1958), which is destroyed by mild alkali

hydrolysis, the serine undergoing β -elimination (Anderson *et al.* 1965). The observation that more serine is destroyed by mild alkali hydrolysis of chondroitin sulphate from old aortae suggests more peptide-polysaccharide linkages in old aortae. The percentage destruction of serine by alkali was lower than that found in the chondroitin sulphate-peptide of cartilage by Anderson *et al.* (1965). Neuberger, Gottschalk & Marshall (1966) observed that the glycosidic serine link is only labile in alkali if both amino and carboxyl groups are involved in peptide linkages. This may not always be the case after papain digestion. It is also possible that the close proximity of the free carboxyl groups of glutamic acid and aspartic acid may interfere with the β -elimination of serine. The keratan sulphate isolated from cornea by Seno *et al.* (1965), which resembled aortic chondroitin sulphate in that it contained more aspartic acid than proline, was also remarkably resistant to alkali hydrolysis. This led these authors to suggest that linkages other than xylosylserine may exist between keratan sulphate and protein in cornea. Alternative linkages may also exist in the aorta.

The evidence discussed above suggests that there are qualitative, as well as quantitative, changes in the glycosaminoglycans of human aortae with age. In particular, it suggests that there are more peptide-polysaccharide linkages in glycosaminoglycans derived from old, rather than young, aortae.

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